Micronucleus frequency in human lymphocytes after exposure to diphenylamine in vitro

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Micronucleus frequency in human lymphocytes after exposure to diphenylamine *in vitro*

Q1 Alfredo Santovito*, Piero Cervella, Massimiliano Delpero

Dipartimento di Biologia Animale e dell'Uomo, University of Turin, Via Accademia Albertina n. 13, 10123 Torino, Italy

ARTICLE INFO

Article history:
Received 7 October 2011
Received in revised form 6 February 2012
Accepted 2 May 2012
Available online xxx

Keywords:
Diphenylamine
DPA
Pesticide
Fungicide
Cytogenetics
Genotoxicity

ABSTRACT

Diphenylamine (DPA) is an antioxidant compound that occurs naturally in several vegetables. It is widely applied in agriculture for preservation of the quality of apples and pears, and used for controlling superficial scald, a disorder that renders fruits of a number of apple cultivars unfit for the market. Because of its anti-oxidative properties, DPA also has several industrial applications. The potential genotoxic effect of DPA on human lymphocytes has previously been investigated in only two studies, which focused on detection of chromosome aberrations and sister chromatid exchange, respectively. In the present analysis, we evaluated micronucleus (MN) formation in freshly isolated human peripheral lymphocytes exposed to different concentrations (0.625, 1.25, 2.50, 5.0 and 10.0 μg/ml) of DPA. Peripheral venous blood was collected from ten healthy subjects, and a total of 10,000 bi-nucleated cells were analyzed. Results indicated that DPA significantly increased the micronucleus frequency at concentrations of 1.25 μg/ml and higher. Significant differences in the MN frequency were also found between the lower dose (0.625 μg/ml) and all other doses tested, with the exception of 1.25 μg/ml. Our results indicate a potential cytogenetic effect of DPA on human cells *in vitro* and require further *in vivo* studies to clarify the actual genotoxicity of this compound and the consequent risks for human health.

1. Introduction

Diphenylamine (DPA) is an antioxidant widely used for preservation of the quality and prevention of post-harvest deterioration of apples and pears, in particular for controlling superficial scald, a disorder that renders fruits of a number of apple cultivars unfit for the market. Because of its anti-oxidative properties, DPA also has several industrial applications, for example as a precursor of dyes, pharmaceuticals, photographic chemicals, and as a stabilizer in nitrocellulose-containing explosives and propellants (reviewed in [1]). Moreover, DPA is a natural compound found in some vegetables, such as onions [2], baked potatoes [3], buckwheat flour [4], black and green tea [5], and dried plums [6].

Eco-toxicological studies indicate that DPA and its derivatives are contaminants in soil and water, and potentially hazardous to aquatic organisms [1]. DPA treatment in short-time studies and during long-term expositions in animal experiments showed an increase of liver- and kidney-damaging effects [7–9]. Lorenzin [10] assessed the amount of pesticide residues in Italian pre-packed meals: DPA was shown to be one of the pesticides most frequently found in fruit and side dishes. Cytogenetic monitoring of human populations exposed to pesticides is generally based on the analysis of the genotoxic effects induced by chronic low doses of complex chemical mixtures. As a consequence, it is often very difficult to associate the observed genotoxic damage to specific chemical classes or compounds. On the other hand, *in vitro* and *in vivo* studies focusing on single substances provide more informative evidence about the genotoxic effects of specific pesticides.

Unfortunately, *in vitro* experiments with DPA are scanty. Dolara and co-workers [11,12] tested the effects of a mixture of 15 pesticides commonly found in Italian foods (including DPA at 14.4%) on human lymphocytes. Results indicated that the pesticide mixture did not induce significant variations in chromosome number or the frequency of chromatid aberrations. Conversely, a moderate but statistically significant increase of sister-chromatid exchange (SCE) at low concentrations of the pesticide mixture was observed. Subsequently, Ardito et al. [13], analyzing the effects of DPA on human lymphocytes, observed a significant increase of SCE frequency only at high concentrations.

In a FAO-WHO joint meeting [14], on the basis of negative results from studies conducted *in vivo* on different animals (Korolev et al., 1976; Gorecka-Turska et al., 1983; Mobil Oil Corp., 1987), it was concluded that although DPA may have genotoxic potential, it is unlikely to be a human genotoxic hazard. In the same meeting, the recommended maximum residue limit (MRL) on apples was 5 mg/kg, while the acceptable daily intake (ADI) for humans for this compound was set at 0–0.08 mg/kg bw [14,25].

* Corresponding author. Tel.: +39 0116704554; fax: +39 0116704508.
E-mail address: alfredo.santovito@unito.it (A. Santovito).

1383-5718/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.mrgentox.2012.05.003

In this study we analyzed the effects of different DPA concentrations on human lymphocytes, by use of the micronucleus (MN) assay. Micronuclei are either a-centric chromosome fragments or whole chromosomes left behind during mitotic cell division, and appear as small additional nuclei in the cytoplasm of interphase cells. In contrast to analyses of CA and SCE, which mainly reveal alterations in chromosome structure, the MN assay – in combination with centromere analysis – may allow detection of both clastogenicity (chromosome breakage) and aneugenicity (chromosome lagging due to dysfunction of the mitotic apparatus) [15–18].

The aim of the study was to determine whether DPA, at different concentrations, could induce genotoxic damage in cultured human lymphocytes. We tested 0.625, 1.25, 2.5, 5 and 10 μg/ml of DPA, where 0.625, 1.25, and 2.5 μg/ml represent the sub-multiple and 10 μg/ml the multiple of the MRL, which is the value accepted for apples (5 μg/ml).

2. Materials and methods

2.1. Chemicals, media, and enzymes

Diphenylamine (DPA) (IUPAC name, *N*-phenylbenzenamine; CAS nr 122-39-4) was obtained from Labservices, Bologna, Italy and dissolved in DMSO (CAS no. 67-68-5). Gibco RPMI 1640 cell-culture medium supplemented with L-glutamine, foetal calf serum, phytohaemagglutinin (PHA), and antibiotics were purchased from Invitrogen-Life Technologies, Milan, Italy. Cytochalasin-B and mitomycin-C (MMC) were obtained from Sigma–Aldrich, Milan, Italy. Methanol, acetic acid, Giemsa staining solution, and conventional microscope slides were purchased from Carlo Erba Reagenti, Milan, Italy. Potassium chloride (KCl) and Sörensen buffer were obtained from Merck S.p.A., Milan, Italy. Vacutainer blood-collection tubes were from Terumo Europe, Rome, Italy. Distilled water was used throughout the experiments.

2.2. Lymphocyte cultures

Peripheral venous blood was collected from ten healthy subjects (mean age ± S.E., 32.90 ± 1.84 years, range 23–40 years), non-smokers, non-drinkers, not under drug therapy, and with no recent history of exposure to mutagens. Informed consent was obtained from all blood donors. The study was approved by the local ethics committee and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

2.3. Blood-sample collection

Heparinized blood samples were obtained by venipuncture and collected in heparinised tubes, for genotoxicity testing. All blood samples were coded, cooled (4 °C), and processed within 2 h after collection.

2.4. Cytokinesis-block micronucleus assay

Heparinized venous blood (0.3 ml) was cultured in 25-cm^2 flasks in 4.7 ml of RPMI-1640 medium supplemented with 20% foetal calf serum (FCS), 2% of the mitogen phytohaemagglutinin (PHA), L-glutamine (2 mM), antibiotics (100 IU/ml penicillin, and 100 μg/ml streptomycin). The cultures were incubated for 72 h at 37 °C, under 5% of CO_2 in the air in a humidified atmosphere.

After 24 h of incubation, DPA at concentrations of 0.625 μg/ml, 1.25 μg/ml, 2.5 μg/ml, 5 μg/ml and 10 μg/ml were added to the cultures. Three control cultures were included: (1) positive control, by adding mitomycin-C (MMC, final concentration 0.01 μg/ml culture); (2) solvent control, 1% DMSO; (3) negative control, culture without DPA or DMSO. After 44 h of incubation, cytochalasin-B was added to the cultures at a concentration of 6 μg/ml to block cytokinesis. Following a further incubation during 28 h at 37 °C, the cells were collected by centrifugation and treated for three min with a pre-warmed mild hypotonic solution (75 mM KCl). After centrifugation and removal of the supernatant, the cells were fixed with a fresh mixture of methanol/acetic acid (3:1, v/v). The treatment with the fixative was repeated three times. Finally the supernatant was discarded and the pellet, suspended in a minimal volume of fixative, was placed on the slides to detect MN by conventional staining with 5% Giemsa (pH 6.8) prepared in Sörensen buffer. Microscope analysis was performed at 1000× magnification with a light microscope (Dialux 20, Leica, Germany). Micronuclei were scored in 1000 bi-nucleated lymphocytes with well-preserved cytoplasm per subject (total 10,000 bi-nucleated cells per concentration), following the established criteria for evaluation of MN (Fenech et al., 2003). A total of 1000 lymphocytes per donor (total 10,000 lymphocytes) were scored to evaluate the percentage of cells with 1–4 nuclei. The cytokinesis-block proliferation index (CBPI) was calculated, according to [19].

2.5. Statistical analysis

Comparison of mean values of the percentage of cells with MN and the CBPI at different exposure conditions and their controls was done by use of the Kruskal–Wallis test. Statistical calculations were carried out with the SYSTAT software package program (version 10.0, Inc., Chicago, IL, USA). All *P*-values were two tailed, and *P*-values of 0.05 or less were considered to correspond with statistical significance for all tests carried out.

3. Results

To assess the effects of DPA on human lymphocytes, the MN frequency was investigated at different concentrations of the pesticide (Table 1). The results indicate that DPA significantly ($P < 0.05$) increased the MN formation compared with the negative control, at all concentrations (Table 1). When compared with the solvent control (1% DMSO), DPA significantly increased MN frequency at all concentrations with the exception of 0.625 μg/ml. Moreover, highly significant ($P < 0.001$) differences in MN frequency were also found between the lowest DPA dose (0.625 μg/ml) and all other concentrations except 1.25 μg/ml. The solvent-control cultures did not show any difference with the negative controls confirming that, at this low concentration (1%), DMSO has no biological effects. The cultures treated with the mutagen MMC showed a consistently increased MN formation compared with the negative and solvent controls. Finally, we observed a decrease, although not statistically significant, of the CBPI values at different DPA exposure levels.

4. Discussion

A large body of literature has been devoted to the analysis of possible toxic effects of pesticides with different animal models and cellular systems. Although various experimental data have provided convincing evidence that pesticides can exert mutagenic/genotoxic effects *in vivo* and *in vitro*, detailed information about aneugenic and clastogenic effects of exposure to specific pesticides is limited and sometimes inconsistent. Among the cytogenetic end-points (CA, SCE, MN) commonly used to investigate the genotoxicity of chemicals, the analysis of MN has often been preferred due to its reliability and sensitivity of MN as a marker of cytogenetic damage [18]. Moreover, the formation of MN after exposure to various pesticides has been reported in several test systems [18]. In this study, we chose the MN approach to evaluate the potential risk for chromosome damage in cultured human lymphocytes exposed to different concentrations of DPA. The importance of DPA in preventing superficial scald of apples, which has serious economic and logistical effects on storage, marketing and distribution of high-quality fruit, is widely recognised, but the available data on the genotoxic potential of DPA are scanty.

Our results show that DPA induces significant micronucleus formation in cells treated with concentrations ≥1.25 μg/ml, including the concentration of 5 μg/ml accepted as MRL for DPA by the WHO and the European Commission. Compared with the SCE analyses performed by Ardito et al. [13], where the cytogenetic damage caused by DPA was significant only at the highest concentration tested (6 μg/ml), we observed a significant increase of MN frequency also at lower concentrations.

These discordant results confirm the higher sensitivity of the MN assay in detecting genotoxic effects related to pesticide exposure. Indeed, while the SCE analysis can only detect alterations in the chromosome structure, formation of MN reveals changes in either chromosome number or chromosome structure, providing a method able to evaluate potential clastogenic and aneuploidogenic exposure simultaneously (Bolognesi et al., 2003). Aneugenic chemicals induce alterations of centromeric DNA [16], with leads to failure of attaching the damaged chromosomes to the mitotic spindle [17], and subsequent formation of MN.

Please cite this article in press as: A. Santovito, et al., Micronucleus frequency in human lymphocytes after exposure to diphenylamine *in vitro*, Mutat. Res.: Genet. Toxicol. Environ. Mutagen. (2012), http://dx.doi.org/10.1016/j.mrgentox.2012.05.003

Table 1
Induction of micronulcei produced by diphenylamine in human lymphocytes *in vitro*.

Test substance	Treatment		BNCs scored	Distribution of BNCs according to the no. of MN				MNs	MN/cell (%) ± S.E.	CBPI ± S.E.
	Period (h)	Dose (μg/ml)		1	2	3	4			
Negative control	48	–	10,000	56	5	0	0	61	0.61 ± 0.14	2.30 ± 0.21
1% DMSO	48	–	10,000	65	7	0	0	72	0.72 ± 0.16	2.17 ± 0.14
MMC	48	0.10	10,000	213	35	4	0	252	2.52 ± 0.51[a]	1.57 ± 0.13
DPA	48	0.625	10,000	79	9	0	0	88	0.88 ± 0.19	1.84 ± 0.07
DPA	48	1.25	10,000	98	11	0	0	109	1.09 ± 0.24[b]	1.76 ± 0.03
DPA	48	2.50	10,000	139	25	1	0	165	1.65 ± 0.33[a]	1.73 ± 0.03
DPA	48	5.00	10,000	145	24	1	0	170	1.70 ± 0.35[a]	1.68 ± 0.06
DPA	48	10.0	10,000	181	21	1	0	203	2.03 ± 0.44[a]	1.59 ± 0.12

BNCs, bi-nucleated cells; MMC, mitomycin-C; DPA, diphenylamine (in 1% DMSO, final concentration in culture). S.E., standard error. CBPI, cytokinesis-block proliferation index = [1 × N_1] + [2 × N_2] + [3 × (N_3 + N_4)]/N, where N_1–N_4 represent the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored.
[a] Significantly different from the solvent control (1% DMSO) $P = 0.000$.
[b] Significantly different from the solvent control (1% DMSO) $P = 0.033$.

Finally, a significant reduction of the CBPI value in cultures treated with DPA was not observed, indicating that DPA does not seem to produce effects on the proliferation/mitotic index when its concentration is equal or less than 10 μg/ml. This result is congruent with those obtained by Ardito et al. [13] for DPA and with data about other pesticides [19,20].

In conclusion, this study shows that exposure of cultured human lymphocytes to DPA results in chromosome damage that leads to an increased frequency of MN. These positive *in vitro* results must be followed-up by *in vivo* studies to clarify the actual genotoxicity of the molecule and consequent risks for human health. To date, DPA has been tested for potential carcinogenicity in few long-term bioassays, where it has not been found carcinogenic in experimental animal models [14]. Moreover, Yoshida and co-workers (1989) have calculated a quite high (111 mg/kg/day) no observable effect level (NOEL) in rats.

In this perspective, it would be useful and interesting to further investigate DPA levels in tissues or blood plasma of people handling and consuming treated fruit in order to correctly assess the potential genotoxic effects of DPA for humans *in vivo*. In fact, the concentration *in vivo* will presumably be much lower and a function of the quantity consumed, plus a dilution factor due to absorption and metabolism.

Conflict of interests

The authors declare that they have no conflict of interests.

Q4 Uncited references

[21–24,26–28].

Acknowledgements

We are grateful to Natascha Rogge for her valuable help and criticism and to all volunteers who participated in this study. The authors are also thankful to the anonymous referees for their helpful comments and constructive suggestions. This work was supported by grants from the Italian Ministry of University and Scientific Research.

References

[1] O. Drzyzga, Diphenylamine and derivatives in the environment: a review, Chemosphere 53 (2003) 809–818.
[2] M.S. Karawya, S.M. Abdel Wahab, M.M. El-Olemy, N.M. Farrag, Diphenylamine, an antihyperglycemic agent from onion and tea, J. Nat. Prod. 47 (1984) 775–780.
[3] E.C. Coleman, C.T. Ho, S.S. Chang, Isolation and identification of volatile compounds from baked potatoes, J. Agric. Food Chem. 29 (1) (1981) 42–48.
[4] I. Yajima, T. Yanai, M. Nakamura, H. Sakakibara, H. Uchida, K. Hayashi, Volatile flavor compounds of boiled buckwheat flour, Agric. Biol. Chem. 47 (4) (1983) 729–738.
[5] M. Nose, Y. Nakatani, T. Yamanishi, Studies on the flavor of green tea. Part IX. Identification and composition of intermediate and high boiling constituents in green tea flavour, Agric. Biol. Chem. 35 (1971) 261–271.
[6] M. Moutounet, P. Dubois, C. Jouret, Major volatile compounds in dried plum, C. R. Acad. Agric. France 61 (10) (1975) 581–585.
[7] F. Alvarez, F.J. Diaz-Alferez, P.J. Perez-Munoz, F. Arteaga-Serrano, A. Martin-Rodriguez, Cystic kidney disease induced by diphenylamine in rats, Actas Urol. Esp. 11 (1987) 656–659.
[8] S.D. Lenz, W.W. Carlton, Diphenylamine-induced renal pappilary necrosis and necrosis of the pars recta in laboratory rodents, Vet. Pathol. 27 (1990) 171–178.
[9] Y. Masubuchi, S. Yamada, T. Horie, Possible mechanism of hepatocyte injury induced by diphenylamine and its structurally related nonsteroidal anti-inflammatory drugs, J. Pharmacol. Exp. Ther. 292 (2000) 982–987.
[10] M. Lorenzin, Pesticide residues in Italian ready-meals and dietary intake estimation, J. Environ. Sci. Health B 42 (7) (2007) 823–833.
[11] P. Dolara, A. Vezzani, G. Caderni, C. Coppi, F. Torricelli, Genetic toxicity of a mixture of fifteen pesticides commonly found in the Italian diet, Cell. Biol. Toxicol. 9 (4) (1993) 333–343.
[12] P. Dolara, F. Torricelli, N. Antonelli, Cytogenetic effects on human lymphocytes of a mixture of fifteen pesticides commonly used in Italy, Mutat. Res. 325 (1) (1994) 47–51.
[13] G. Ardito, B. Bramanti, M.P. Bigatti, L. Lamberti, P. Dolara, Cytogenetic effect of thiabendazole and diphenylamine on cultured human lymphocytes: sister chromatid exchanges and cell cycle delay, Boll. Soc. Ital. Biol. Sper. 72 (1996) 171–178.
[14] Joint FAO/WHO, Meeting on Pesticide Residues, JMPR Publications, 1998. http://www.inchem.org/documents/jmpr/jmpmono/v098pr07.htm.
[15] A. Elhajouji, M. Cunha, M. Kirsch-Volders, Spindle poisons can induce polyploidy by mitotic slippage and micronucleate mononucleates in the cytokinesis-block assay, Mutagenesis 13 (1998) 193–198.
[16] M. Fenech, The *in vitro* micronucleus technique, Mutat. Res. 455 (2000) 81–95.
[17] E.M. Parry, J.M. Parry, C. Corso, A. Doherty, F. Haddad, T.F. Hermine, G. Johnson, M. Kayani, E. Quick, T. Warr, J. Williamson, Detection and characterization of mechanisms of action of aneugenic chemicals, Mutagenesis 17 (6) (2002) 509–521.
[18] C. Bolognesi, Genotoxicity of pesticides: a review of human biomonitoring studies, Mutat. Res. 543 (2003) 251–272.
[19] D. Yüzbaşioğlu, M. Celik, S. Yilmaz, F. Unal, H. Aksoy, Clastogenicity of the fungicide afugan in cultured human lymphocytes, Mutat. Res. 604 (1–2) (2006) 53–59.
[20] A. Santovito, P. Cervella, M. Delpero, *In vitro* aneugenic effects of the fungicide thiabendazole evaluated in human lymphocytes by the micronucleus assay, Arch. Toxicol. 85 (6) (2011) 689–693.
[21] M.C. Alavanja, M.H. Ward, P. Reynolds, Carcinogenicity of agricultural pesticides in adults and children, J. Agromed. 12 (2007) 39–56.
[22] M. Çelik, F. Ünal, D. Yüzbaşioğlu, M.A. Ergün, O. Arslan, R. Kasap, *In vitro* effect of karathane LC (dinocap) on human lymphocytes, Mutagenesis 20 (2004) 101–104.
[23] T. Clary, B. Ritz, Pancreatic cancer mortality and organochlorine pesticide exposure in California 1989–1996, Am. J. Ind. Med. 43 (2003) 306–313.
[24] J.M. Garcia-Sagredo, Fifty years of cytogenetics: a parallel view of the evolution of cytogenetics and genotoxicology, Biochim. Biophys. Acta 1779 (2008) 363–375.

Please cite this article in press as: A. Santovito, et al., Micronucleus frequency in human lymphocytes after exposure to diphenylamine *in vitro*, Mutat. Res.: Genet. Toxicol. Environ. Mutagen. (2012), http://dx.doi.org/10.1016/j.mrgentox.2012.05.003

[25] Joint FAO/WHO, Meeting on Pesticide Residues, JMPR Publications, 1984. http://www.inchem.org/documents/jmpr/jmpmono/v84pr16.htm.
[26] G.L. Poletta, A. Larriera, E. Kleinsorge, M.D. Mudry, Genotoxicity of the herbicide formulation roundup (glyphosate) in broad-snouted caiman (Caiman latirostris) evidenced by the Comet assay and the micronucleus test, Mutat. Res. 672 (2) (2009) 95–102.
[27] M. Watanabe-Akanuma, T. Ohta, Y.F. Sasaki, A novel genotoxic aspect of thiabendazole as a photomutagen in bacteria and cultured human cells, Toxicol. Lett. 158 (2005) 213–219.
[28] L.R. Webster, G.H. McKenzie, H.T. Moriarty, Organophosphate-based pesticides and genetic damage implicated in bladder cancer, Cancer Genet. Cytogenet. 133 (2002) 112–117.

Please cite this article in press as: A. Santovito, et al., Micronucleus frequency in human lymphocytes after exposure to diphenylamine *in vitro*, Mutat. Res.: Genet. Toxicol. Environ. Mutagen. (2012), http://dx.doi.org/10.1016/j.mrgentox.2012.05.003